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ORIGINAL ARTICLE

The influence of melatonin on growth of *E. coli* O157:H7 in pure culture and exogenous melatonin on faecal shedding of *E. coli* O157:H7 in experimentally infected wethers*

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Abstract

Aims: To determine if exogenous melatonin (MEL) influences growth of *Escherichia coli* O157:H7 in pure culture and if MEL affects faecal shedding patterns of *E. coli* O157:H7 or total leucocyte counts in sheep.

Methods and Results: Two strains of *E. coli* O157:H7 were cultured in the presence of varying concentrations of MEL. Maximal specific growth rates of *E.coli* O157:H7 strains were not affected by MEL addition in pure culture. Wethers ($n = 16$) received either 0 (CONT) or 25 mg MEL hd^{-1} day^{-1} for 21 days. Daily shedding patterns of *E. coli* O157:H7 were not different ($P > 0{\cdot}10$) between groups with faecal populations of *E. coli* O157:H7 decreasing daily ($P < 0{\cdot}01$) in both groups. However, shedding tended to differ between the control and treated group by the end of the experiment. Total WBC and differential leucocyte counts were not affected by treatment.

Conclusions: Melatonin had no affect on specific growth rates in pure culture nor did the administration of exogenous MEL alter bacterial shedding patterns or immune response indicators in experimentally infected wethers exposed to a long photoperiod.

Significance and Impact of the Study: Although MEL did not affect shedding patterns or gastrointestinal populations of *E. coli* O157:H7, the tendency for MEL-treated sheep to shed less *E. coli* O157:H7 towards the end of the experiment warrants further research. Providing MEL for a longer period of time, or at greater concentrations, may elucidate a potential role that MEL plays in the seasonal shedding patterns of *E. coli* O157:H7 in livestock.

Introduction

Escherichia coli O157:H7 is responsible for a number of human foodborne illnesses in the USA (0·45% of all foodborne illnesses) (Animal and Plant Health Inspection Service 2001) with a majority of these cases reported during the warmer months of the year (Rangel *et al.* 2005). Coincidentally, a greater prevalence of *E. coli* O157:H7 shedding by dairy and beef cattle is exhibited during the summer and spring months (Barkocy-Gallagher *et al.* 2003; Miller *et al.* 2003) when ambient temperature, relative humidity, and day length (photoperiod) are increased. However, research has indicated little to no effect of heat stress on daily faecal shedding patterns of *E. coli* O157:H7 (Fitzgerald *et al.* 2003; Edrington *et al.* 2004). Despite trends of increased shedding of *E. coli* O157:H7 during the summer months, to our knowledge, research has yet to examine the effects of photoperiod or hormones associated with daylength on the prevalence of *E. coli* O157:H7.

The pineal gland is an organ which responds to photoperiod and demonstrates circannual rhythms in hormone synthesis and secretion (Bubenik *et al.* 1998). Melatonin (MEL) is secreted by the pineal gland and is available to all tissues and organs, is widely distributed in every cell compartment, and is present in bacteria, protists, plants and vertebrates (Hattori *et al.* 1995; Manchester *et al.* 1995). Circadian rhythms of serum MEL are characterized by increased synthesis and secretion during the dark period of the light/dark cycle with the duration of nocturnal MEL production proportional to the length of the night (Pévet 2003). In addition to its diurnal rhythm, MEL also exhibits seasonal cycles in response to daylength and may play a role in the regulation of annual rhythms of physiological functions and behaviour in mammals (Pévet 2003). Literature profiling circannual serum MEL concentrations has indicated total serum MEL levels only vary slightly between seasons but the duration of peak MEL concentrations differ with the change in daylength (Pévet 2003). Alila-Johansson *et al.* (2001) monitored seasonal variation of endogenous serum MEL in goats and found concentrations and the duration of high serum levels were greatest during winter.

Extrapineal sources of MEL have also been reported in the retina, Hardarian gland, and the gastrointestinal tract (GIT), with concentrations in the digestive tissues substantially surpassing levels in the peripheral blood (Bubenik 2002) as well as exceeding pineal MEL by more than 400 times (Huether 1993). Mice exposed to a light/dark cycle had higher jejunal tissue MEL concentrations during the scotophase with no differences in stomach tissue MEL concentrations observed between the photo- and scotophase (Bubenik *et al.* 1993). Changes in GIT MEL levels have also been linked with feed intake. Pigs fasted for 30 h had higher MEL concentrations in most GIT tissues (except the rectum) following refeeding with higher levels detected in the distal segments of the GIT (Bubenik *et al.* 1996). MEL has also been detected in the luminal fluid and mucosa of the bovine (and porcine) with concentrations exceeding that found in the serum (Bubenik *et al.* 1999).

Melatonin also plays a role in the antioxidative defence system by acting as a scavenger of free radicals (Tan *et al.* 2000). Decreased circulating MEL levels have been linked to a higher risk of breast cancer, cardiovascular disease, reproductive difficulties, and gastrointestinal discomfort in women working night shifts, who are exposed to longer periods of light (Biovin and James 2002; Schernhammer and Hankinson 2005). Mice exposed to short day lengths had higher lymphocyte numbers and macrophage counts as compared with those exposed to a long photoperiod (Brainard *et al.* 1987; Yellon *et al.* 2005). Total white blood cell counts (Blom *et al.* 1994) have been enhanced in deer mice exposed to short day lengths. Minton *et al.* (1991) observed lambs under a constant lighting regimen had decreased pineal melatonin and found they exhibited lower total leucocytes ($9{\cdot}2 \times 10^3$ μl^{-1}) compared with those exposed to a photoperiod (12L:12D) in which diurnal rhythms were evident ($11{\cdot}4 \times 10^3$ μl^{-1}). MEL provided to pigs consuming a diet to increase the occurrence of gastric ulcers decreased the severity of those ulcers when MEL was provided in graded levels (Ayles *et al.* 1996) thus illustrating MEL may play a role in nonspecific immunity.

Prevalence of *E. coli* O157:H7 is highest when the duration of peak serum MEL levels are shortest, but decreases to nearly undetectable levels when the duration of peak serum MEL levels is longest. This potential relationship led us to investigate: (i) the effects of varying concentrations of MEL on two strains of *E. coli* O157:H7 in pure culture and (ii) the effects of exogenous MEL on daily faecal shedding and GIT populations of *E. coli* O157:H7 in experimentally infected sheep.

Materials and methods

E. coli O157:H7 strains and chemicals

Escherichia coli O157:H7 strain 933 was obtained from the American Type Culture Collection (Manassas, VA; ATCC 43895). Strain 6058, which was isolated from ground beef following a fatal outbreak of haemorrhagic colitis, and 2336 were obtained from Dr Dan Rice (Field Disease Investigation Unit at Washington State University, Pullman). Strains 933 and 6058 were made resistant to 20 μg ml^{-1} nalidixic acid (NA) and 25 μg ml^{-1} novobiocin (NO) while strain 2336 was made resistant to 20 μg ml^{-1} rifampicin. All reagents and chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) and all media were purchased from Difco Laboratories (Sparks, MD, USA).

Pure culture experiments

Pure cultures of *E. coli* O157:H7 strains 933 and 6058 were individually added (0·5 ml each) to 9·0 ml autoclaved tryptic soy broth (TSB). MEL solutions, made the day of each experiment, were added to achieve final concentrations of 0·0, 0·43, 0·86, 1·72, 3·44, and 6·88 pmol ml^{-1}. Treatment levels were based on average serum concentrations in sheep, during the photophase, as previously reported (Kennaway *et al.* 1982). Culture tubes were sealed, vortexed, and incubated at 39°C. Optical densities (OD) were recorded every 30 min during growth in TSB using a 20D spectrophotometer (Thermo Electron, Rochester, NY, USA) until a maximum OD of

0·6 was attained. The maximal specific growth rate (MSGR) was calculated during the exponential growth phase using the formula (ln OD2 − ln OD1)/Δt.

Sheep experiment

Mature crossbred wethers ($n = 16$; avg. BW 53·4 ± 7·4 kg) were assigned randomly to one of two groups: control (CONT) or 25 mg MEL hd^{-1} day^{-1} (MEL; Sigma Chemical Co.) with all procedures pre-approved by the Animal Care and Use Committee of the USDA-ARS, Southern Plains Agricultural Research Center. Animals were housed in isolation rooms with four sheep per room, two rooms per treatment and were acclimated for 7 days to a photoperiod consisting of 16 h light and 8 h dark (16L : 8D). Following the acclimation period, wethers were orally dosed for 21 days (days 0–21) with gelatin capsules (no. 000; PCCA, Houston, TX, USA) containing either ground alfalfa or 25 mg MEL plus ground alfalfa for CONT- and MEL-treated animals respectively. Wethers had *ad libitum* access to water, commercial sheep pellets, and bermudagrass hay. Seven days (day 7) following the initial MEL treatment, all animals were experimentally infected with *E. coli* O157:H7 strain 2336 (10 ml; $6{\cdot}4 \times 10^8$ CFU ml^{-1} per sheep) via oral gavage. To monitor shedding patterns of the experimental strain, faecal samples were collected daily via rectal palpation on days 8–21. Faecal material (1 g) was serially diluted in phosphate-buffered saline (PBS), plated on MacConkey agar containing 20 μg rifampicin ml^{-1} and incubated (37°C) overnight. Sheep were humanely euthanized on day 21 (14 days postinoculation) using a commercial euthanizing agent (Euthasol®; Virbac AH, Inc., Ft Worth, TX, USA), to evaluate gut populations of the experimental strain. Luminal contents (30–50 ml) from the rumen, ileum, caecum and rectum were collected, serially diluted, and plated as above.

Blood was collected via jugular venipuncture on days 7, 14, and 21 to determine total WBC and differential leucocyte counts. Blood was collected in 5 ml heparinized vaccutainer tubes. To determine total WBC counts, blood cells were lysed with ultra-pure water, washed with Hanks' balanced salt solution (HBSS), centrifuged at 1200 ***g*** for 15 min, and counted using a haemacytometer. For leucocyte differential counts of monocytes, lymphocytes, and polymorphonuclearcytes (PMN), approximately 10 μl blood was smeared on pre-cleaned microscope slides, stained, and counted within 5 days of the collection time. For the analysis of MEL, weekly blood samples were collected from all animals 2 h following the introduction of light. Serum MEL was quantified by RIA using a commercial kit according to manufacturer's specifications (Buhlmann MEL direct RIA; American Laboratory Products Company, Windham, NH, USA).

Statistical analysis

Specific growth rates of *E. coli* O157:H7 strains 933 and 6058 in pure culture were analysed using the GLM procedure of SAS (2001). Pure culture experiments were conducted in triplicate and data pooled for analysis. Daily faecal shedding patterns were analysed as repeated measures using the MIXED procedure of SAS. White blood cells, differential leucocyte counts, and luminal *E. coli* populations were analysed using the GLM procedure of SAS. All data are reported as least squares means. Effects with *P*-values less than 0·05 were declared significant.

Results

Pure culture experiments

The specific growth rate (h^{-1}) of each tested strain (933 and 6058), when grown in TSB medium, were not affected ($P > 0{\cdot}1$) by the addition of MEL regardless of concentration (data not shown).

Sheep experiment

Faecal swabs taken prior to inoculation demonstrated animals were not shedding *E. coli* O157:H7 or *E. coli* O157:H7 capable of growth on rifampicin supplemented MacConkey's agar (data not shown). Daily faecal shedding patterns (Fig. 1) of the experimental strain were similar ($P = 0{\cdot}37$) between the CON and treated group with shedding decreasing daily ($P < 0{\cdot}01$) over the 14 days in both groups. Initial shedding of *E. coli* O157:H7 was

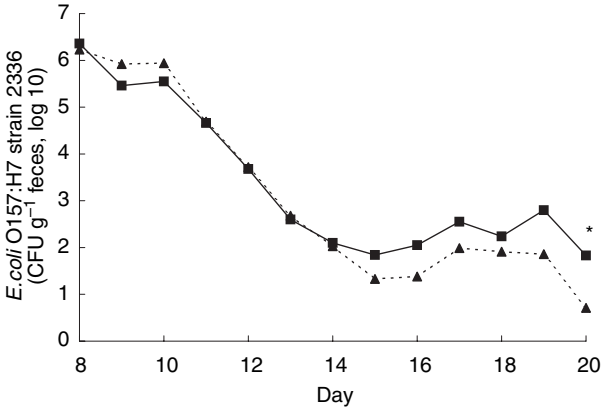

Figure 1 Faecal shedding of *Escherichia coli* O157:H7 by sheep receiving either 0·0 (■) or 25·0 (▲) mg MEL hd^{-1} day^{-1} for 21 days. $*P = 0{\cdot}05$.

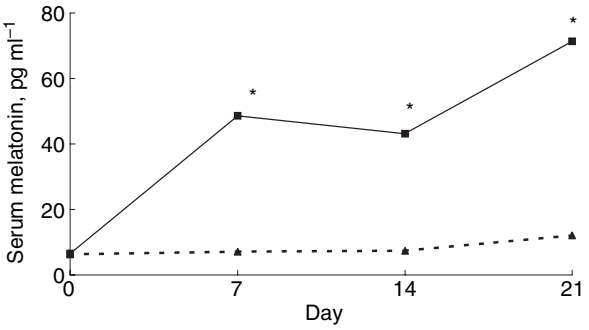

Figure 2 Serum melatonin (MEL) concentrations of sheep receiving 0·0 (■) or 25·0 (▲) mg MEL hd^{-1} day^{-1} for 21 days. Differences ($P < 0·05$) in MEL concentrations are indicated by (*).

similar on day 8, with 6·36 and 6·23 CFU g^{-1} faeces ($\log_{10}$) for CON and MEL-treated animals respectively. By day 20, bacterial counts were 1·83 and 0·71 CFU g^{-1} faeces ($\log_{10}$) for CON and MEL-treated animals respectively. Control sheep tended to, or shed more, *E. coli* O157:H7 on day 19 ($P = 0·09$) and day 20 ($P = 0·05$) than those receiving MEL. However, bacterial counts from rectal samples taken on day 21 were similar ($P = 0·68$) between the CON and treated group.

Similar to daily faecal shedding patterns, no differences ($P > 0·10$) were observed in the populations of *E. coli* O157:H7 in ileal, caecal, or rectal contents. The experimental strain was not isolated from the rumen contents of any sheep.

To evaluate the immune response indicators for the animals, total WBC and differential leucocyte counts were analysed on days 7, 14, and 21 of the experiment. Although neither treatment, nor treatment × day, effects were observed, total WBC counts decreased over the course of the study ($P < 0·01$) in all animals. On day 7 (prior to inoculation with *E. coli* O157:H7), WBC counts were $1·5 \times 10^4$ and $1·0 \times 10^4$ for CON and MEL sheep, respectively, decreasing one log by day 21. Differential leucocyte counts were also similar between the CON and treated group with no day or treatment × day effect observed.

Serum concentrations of MEL (Fig. 2) differed between groups ($P < 0·01$). Initial serum MEL concentrations were 6·1 and 6·5 pg ml^{-1} for CON and MEL sheep respectively. However, by the end of the experiment (day 21), average serum MEL concentrations were 12·1 pg ml^{-1} for CON sheep compared with 71·3 pg ml^{-1} for those receiving MEL.

Discussion

Ruminants serve as reservoirs of *E. coli* O157:H7 strains capable of producing disease in humans. The majority of outbreaks, for which a source has been identified, have been linked to the consumption of undercooked beef (Dean-Nystrom *et al.* 1999). The Centers for Disease Control and Prevention (Rangel *et al.* 2005) estimate 73 000 cases of infection and 61 deaths occur in the USA yearly with outbreaks tending to peak during the warmer months of the year. Seasonal changes in faecal populations of *E. coli* O157:H7 by various livestock species have also been observed with the greatest prevalence exhibited during the spring and summer months (Barkocy-Gallagher *et al.* 2003; Miller *et al.* 2003).

Circannual rhythms of MEL, differentiated by duration time of peak serum concentrations, have been thought to play a role in the regulation of annual rhythms of physiological functions and behaviour in mammals (Pévet 2003). Seasonal variation in serum MEL profiles has been associated with immunity (Pévet 2003), reproductive rhythms of sheep (Kennaway *et al.* 1982; Barrell *et al.* 2000), goats (Alila-Johansson *et al.* 2001), and deer (García *et al.* 2003), and milk production in dairy cattle (Dahl *et al.* 2000). Serum profiles are characterized by an increase in the duration of peak MEL levels during winter months with a shorter duration of peak MEL exhibited during long days (Alila-Johansson *et al.* 2001; Pévet 2003). Coincidentally, the duration of peak serum MEL concentrations is longest when shedding of *E. coli* O157:H7 is decreased, but is shorter in length when *E. coli* O157:H7 is most prevalent, which may suggest MEL plays a part, either directly or indirectly, in the seasonal shedding patterns of *E. coli* O157:H7.

A direct effect of MEL on growth of *E. coli* O157:H7 was not evident in our pure culture studies. No change in growth rates of either *E. coli* O157:H7 strain was observed when exposed to varying concentrations of MEL. MEL concentrations used in this experiment were low, but were similar to circulating levels.

Patterns in faecal shedding of *E. coli* O157:H7 strain 2336 were comparable with that observed by Edrington *et al.* (2003) in experimentally infected sheep. Faecal *E. coli* O157:H7 populations at the start of the experiment were initially high but decreased to levels more typical of a naturally infected animal by day 14. Although not statistically significant, a tendency ($P > 0·05$) for decreased shedding of *E. coli* O157:H7 in MEL treated sheep appeared toward the end of the trial (day 15 through day 21). Greater differences in shedding of *E. coli* O157:H7 may have resulted had administration of MEL continued beyond day 21. Edrington *et al.* (2005) observed no differences in shedding of *E. coli* O157:H7 when cattle were treated with a low MEL dose. However, when cattle were given 10× the original dose for a period of 4 days, those receiving MEL shed less *E. coli* O157:H7 than controls.

With an average BW of 53·4 kg, sheep in this experiment received a dose (0·47 mg MEL kg^{-1} BW) similar to the low dose given by Edrington *et al.* (2005). A higher dose, or a longer treatment period, may have produced similar results.

Overall, the health status of animals in this experiment appeared normal. Sheep were handled only as necessary to keep stress to a minimum. Despite the decrease in total WBC, leucocyte numbers were in accordance with that reported by Plumb (1995) for healthy sheep ($4–12 \times 10^3$). Auchtung *et al.* (2004) observed enhanced immune status in dairy cows exposed to a short-day photoperiod with lymphocyte proliferation averaging 197·6 and 326·5% for long-day and short-day photoperiods respectively. Although photoperiod in the current experiment simulated that of a long-day, the photoperiod was similar for all treatment groups. Thus, any possible differences in immune status should be due to treatment.

In the current study, MEL supplementation did not appear to affect faecal shedding patterns or gut populations of *E. coli* O157:H7 in experimentally infected sheep nor did it affect any of the indicators of an immune response. However, the tendency for MEL-treated sheep to shed less *E. coli* O157:H7 towards the end of the experiment warrants further research. Providing MEL at a similar dose but for a longer period of time, and/or at a greater concentration than given in the current study, may provide further information on the importance of MEL and its potential role in the seasonal shedding patterns of *E. coli* O157:H7 in livestock.

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